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Metal surfaces were allowed to become colonized by estuarine bacteria, after which half the samples were subjected to grazing for several days by washed cultures of protozoa. Samples from grazed and non-grazed metal stubs were examined with epifluorescent microscopy for numbers of bacteria on the surfaces, and an analysis of variance test was performed on data to determine significant differences. Bacterial activities on the surfaces of stainless steel 316 in the presence and absence of grazing protozoa was assessed by trapping

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$^{14}CO_2$ metabolized from ^{14}C-glutamic acid in heterotrophic uptake experiments. Appropriate controls were established to account for respiration from residual bacteria remaining with washed protozoa. Data were analyzed by an analysis of variance test. Results showed that grazing significantly increased both the numbers and metabolic activity of bacteria on metal surfaces. Reasons for such results are discussed.

Although results imply that grazing protozoa can indirectly influence corrosion rates, there probably would be no point in pursuing it further with a laboratory approach for reasons discussed in the report. The project provided a foundation for future in situ studies, and the data are being prepared for publication.

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FINAL REPORT TO THE OFFICE OF NAVAL RESEARCH

Title: Effects of Grazing on Bacteria-Mediated Corrosion of Metals in Seawater

ONR Contract No. : N00014-83-0652

Project Period: August 1983-August, 1985

Principal Investigator: Sharon G. Berk

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OBJECTIVES

I. Field Work

The field objective was to obtain isolates of both bacteria and protozoa associated with various types of metals in a natural estuarine environment. These isolates would be cultured and used in the laboratory to address the next two objectives.

II. Effect of Protozoa on Total Numbers of Bacteria on Metals

This objective included testing a couple methods of enumerating bacteria on metal surfaces. The main objective was to determine whether protozoan grazing significantly affected the total numbers of bacteria associated with metal surfaces. Specifically, the objective was to enumerate bacteria on metals after grazing by protozoa had occurred, and to compare these numbers with those on surfaces where grazing was absent. We wished to determine whether grazing activities increased the bacterial numbers as has been shown for bacteria on certain natural substrates. The implication of such findings is that grazing may indirectly affect corrosion rates of metals by affecting the abundance of bacteria on metal surfaces. We later believed that this objective was not as important as the third objective, the effects of grazing on bacterial community respiration, as an indication of effects of grazing on bacterial activities on the surfaces. The total numbers may be decreased by grazing, yet the activities may be higher if grazing removes senescent bacterial cells.

III. Determination of Effects of Grazing on Bacterial Activities

This objective was meant to determine bacterial activities by measuring bacterial community respiration in the presence and absence of protozoan predation. The implication of finding increased respiration in the presence of grazers is the same as that of Objective II., i.e., that grazing may indirectly influence corrosion rates by increasing bacterial activities on metal surfaces. This objective was met by conducting experiments in which radioactive CO_2 from bacterial metabolism of ^{14}C-glutamic acid was trapped and measured with liquid scintillation techniques.

METHODS

Field Work --

Metal coupons (titanium, 316 stainless steel, aluminum/bronze, and 90:10 copper/nickel) were polished with sandpaper and cut to 1.0 x 5 cm pieces. These were washed with 95% ethanol and rinsed with sterile distilled water. They were suspended from the pier at the Chesapeake Biological Laboratory of the University of Maryland (Solomon's,MD) for periods of two weeks. Deployment and retrieval was accomplished by placing the metals in plastic bags in order to prevent them from being covered with organisms from neuston as they passed through the water surface layer. After retrieval, the coupons were shaken lightly in filter-sterilized seawater and placed into 0.1% Cerophyl (dried cereal leaves) media for 24 hours to promote growth of bacteria and protozoa from the metal surfaces. Agar plated containing an estuarine seawater medium supplemented with 0.1% Cerophyl were streaked to isolate bacterial colonies, and protozoa were removed with a micropipet to fresh

Cerophyl medium. Fourteen morphologically distinct strains (colony types) were isolated, and care was taken to ensure that at least two were isolated from each metal. A variety of protozoa were present, however, only two species of hypotrich ciliates were cloned and cultured for several reasons. They were the easiest to raise, the most likely to have a close association with surfaces, and the most likely to be feeding on bacterial films. All cultures of both bacteria and protozoa were maintained routinely in the lab.

At the end of the first year, the principal investigator took a new position at a new institution, the Colorado School of Mines in Colorado, and after several months there, the hypotrich cultures began to decline. Therefore, at a later date new estuarine hypotrich isolates were obtained as described above from metals set out at the EPA's Gulf Breeze, FL lab. At that lab we had the cooperation and assistance of staff members there who set the metals out in estuarine environments for us. Although metals were deployed in three different locations of various salinities, the best results were obtained from only one site near the EPA lab at a salinity of 23 mg/kg. During that sampling trip, bacteria were also isolated from each site, but in a different manner from those isolated earlier at Solomons, MD. The earlier procedure involved placing rinsed metal coupons into bacterial media in small flasks. The problem with that technique was that during the course of incubation, certain metals, particularly the copper/nickel, apparently leached out into the media in concentrations toxic to many bacteria and to all protozoa. Therefore, the new approach was to rinse the stubs with sterile water and to rub them across the surface of agar plates using sterile forceps. Most protozoa were isolated directly by removing

them with sterile micropipets while viewing them through a stereo microscope. In addition, decaying plant material, including Thallasia leaves, was collected to ensure isolation of protozoa associated with surfaces.

Enumeration —

Each of the fourteen original Solomons isolates was tested for determination of the level of sonication they could withstand, in order to evaluate the usefullness of sonication for removal of bacteria attached to surfaces. The bacteria were examined and counted on filters after sonication and after being treated with acriding orange fluorescent dye. Clean metal stubs were then incubated in bacterial cultures for a week. Sonication was then used to see if this procedure would result in a suspension of bacteria which could be counted in an accurate and reproducible manner. A comparison of numbers from sonicated and non-sonicated metal surfaces was made, and indicated that it is not possible to quantitatively remove bacteria from the coupons in this manner. Bacteria were removed from the surfaces only at sonication intensities and durations above those which destroyed many bacterial cells. This was true for all strains of bacteria.

We then tried staining bacteria on stubs with acridine orange and enumerating them by epifluorescence microscopy. This worked well in the experiments, since we used pure cultures of bacteria for most experiments. Acridine orange dye could be placed directly on the stubs, allowed to stand 2 to 3 min, and rinsed with filtered water. They were then allowed to dry prior to being placed on a microscope slide for observation. This procedure is even easier than that for

enumeration of bacteria from water samples.

To find the effect of protozoa on bacterial numbers, cultures of a bacterial isolate from titanium were centrifuged and resuspended in filtered half-strength seawater. Titanium stubs were soaked in 70% ethanol for about an hour, then flame-sterilized with 95% ethanol and rinsed with sterile water to remove any alcohol residue. They were placed into small sterile petri dishes filled with 10 ml of half-strength seawater. To some dishes, approximately 0.1 ml of bacterial suspension was added.

After 4 days of incubation with bacteria, half the stubs were exposed to protozoan grazing. Hypotrichs were collected from cultures in small petri dishes and washed several times by gentle centrifugation in a table-top centrifuge with tapered 10 ml test tubes. The protozoa were repeatedly centrifuged and resuspended in a final dilution of 1:200,000 of the culture media. Stubs which had incubated with bacteria were removed with sterile forceps, rinsed with filter-sterilized seawater, and placed in other sterile containers. To some of these stubs 0.5 ml of the washed hypotrich suspension and 3.0 ml of sterile half-strength seawater were added, while others (controls) received 3.5 ml of the seawater without added protozoa. Other sterile stubs which had not incubated with bacteria were placed in seawater with hypotrichs added to check for numbers of bacteria resulting from residual bacteria associated with the washed hypotrichs.

Protozoa were allowed to graze 4 days, after which stubs were removed, rinsed, and stained with acridine orange dye for enumeration of bacteria on the surfaces. Numbers of bacteria within a grid on 5 fields of stubs from each treatment were determined and recorded. After

subtraction of the numbers of bacteria in the treatment with washed protozoa alone (i.e., to subtract numbers of residual bacteria) from numbers of the treatment consisting of protozoa plus the added attached bacteria, a one-way analysis of variance test (ANOVA) was performed on data from the two treatments to determine significant differences.

Respiration Studies —

Several preliminary heterotrophic uptake experiments were conducted using glass coverslips as the physical substrate in order to determine the concentrations of both bacteria and protozoa to use, as well as the amount of radioactivity to use to see significant metabolism of the radioactive substrate. We also needed to test the duration of incubations. We did not want to sacrifice too many metal coupons or use too much radioactive substrate before we were ready to run the experiments in full with several replicates of metals. The hypotrichs alone were tested to determine the amount of CO_2 produced from the radioactive glutamic acid, and it was found to be negligible, not different from background counts. We were then ready to run similar experiments with metal substrates.

Due to many unexpected problems, each experiment with metals led to new approaches for the next. All bacteria derived from metal surfaces in estuarine water were stored on agar slants of an estuarine medium. Bacteria were grown 24–48 hours in liquid media, after which they were washed by centrifugation and resuspension. Cells were collected in sterile Sorvall tubes, rinsed and resuspended in filter-sterilized half-strength seawater. The seawater was obtained from off the coast of Virginia using large Nalgene carboys.

Metal stubs (made slightly smaller than previous in order to fit

through the neck of a 25 ml Erlenmeyer flask used in the heterotrophic uptake procedures) were cleaned and sterilized as described above for the enumeration studies. For one set of experiments, a bacterial strain, designated 5 SS, isolated from stainless steel, was used in combination with stainless steel stubs. Stubs were placed in the bottom of sterile scintillation vials, and 10 ml of sterile seawater was added, along with 0.1 ml of the bacterial suspension and 0.2 ml of filter-sterilized Cerophyl medium to promote bacterial growth.

The film was allowed to form 5 days before hypotrichs were added. Stubs were removed as described above and placed into sterile scintillation vials containing 3.5 ml seawater. Approximately 900 hypotrichs per vial were added and allowed to graze 4 days. Other stubs prepared without hypotrichs were allowed to incubate 4 days.

Stubs were removed, rinsed, and placed in 5 ml sterile seawater in 25 ml flasks. ^{14}C-glutamic acid was diluted such that 100 ul could be added to the flasks, resulting in 0.24 uCi/flask. Serum stoppers containing plastic cups for sampling the head space were placed over the scintillation vials, and respiration was allowed to proceed for 3.5 hours, followed by injection of a 2N sulfuric acid solution for releasing dissolved CO_2. The flask contents were allowed to stand 20 min, during which time they were shaken by hand occasionally. Phenylethylamine (0.15 ml) was injected through the serum stoppers into the plastic cups to collect the respired CO_2. After 30 min, the phenylethylamine was removed from the cup through the serum stopper with a syringe and injected into 10 ml of Aquasol (ICN) scintillaiton cocktail, and counts per minute (CPM) were determined with a liquid scintillation counter.

For another set of experiments, a similar procedure was performed with the following changes: Sets of stubs were set up with washed protozoa alone (no added bacteria) to subtract respiration of residual bacteria associated with the washed protozoan culture. Also, 4-6 stubs were placed in small plastic petri dishes rather than in scintillation vials for bacterial film formation and grazing to occur, because in several cases, protozoa died in scintillation vials even when vials were new and cleaned.

We thought that allowing the bacterial film to form first in the total absence of protozoa may be too unnatural, since we found that in some experiments the respiration from residual bacteria associated with protozoa in controls (those in which the 5 SS strain was not added) exceeded that of the 5 SS bacteria alone, thus indicating that either time of arrival or bacterial species or both may be very important. Perhaps activities of protozoa <u>during</u> bacterial film formation is important, and may be the reason for such results.

Therefore, we changed the procedure to allow protozoa and bacteria to occur together initially using only bacteria associated with the protozoan cultures. Protozoa were washed by centrifugation, and the supernatant was mixed in a Vortex mixer and passed through a 5 um pore Nitex netting to screen out any remaining hypotrichs (which are ~30-40 um long X 10-15 um wide), but allow bacteria to pass through. Numbers of bacteria in the filtered supernatant and the final hypotrich suspension were enumerated with acridine orange epifluorescent microscopy and adjusted to be equal in all treatments with metals. Bacterial film formation and protozoan grazing interactions were allowed to proceed four days.

RESULTS

The analysis of variance test (ANOVA) showed that numbers of bacteria on the titanium surfaces were significantly greater ($F_{1,8}$ = 5.8, $0.05 > p > 0.025$) in the presence of grazing hypotrichs, even after subtraction of numbers resulting from washed hypotrichs alone. Acridine orange epifluorescent counts of bacteria on metal surfaces worked well for these bacteria allowed to incubate a total of 8 days (4 days prior to addition of protozoa, and 4 days of grazing or additional incubation). Acridine orange preparation of solid flat surfaces was very simple, and easier than enumeration of bacteria from water samples.

In the first set of respiration experiments using bacterial strain 5 SS and stainless steel, the average radioactivity expressed as counts per min (CPM) of the samples exposed to protozoan grazing activities were 163-fold greater than the average CPM of those with bacteria alone. Hypotrichs were washed off the stubs during preparation for incubation with isotopes, so their respiration would not be included in the CPM determinations. In later studies, the bacteria remaining with hypotrichs after on a few washes was found to be only as great as 2-fold that in the supernatant on one occasion, and half that on another occasion. In this first experiment, the hypotrichs were washed to 200,000-fold dilution of the protozoan culture, so the 163-fold higher respiration was probably a real phenomenon due to grazing activities.

In another set of experiments where residual bacterial respiration was considered and subtracted from the respiration of the 5 SS bacteria subjected to grazing, results showed that the average bacterial

respiration (5 SS) was 60-fold higher in the presence of grazers than in their absence. Experiments were done at very different times under slightly different conditions such as numbers of added bacteria, numbers of hypotrichs, condition of hypotrich stock cultures, etc., so we would not expect identical magnitudes of increase. Raw data from these two experiments were pooled and an ANOVA was performed comparing the CPM from metals without the grazing protozoa and those where grazing had occurred. Results showed that the stubs subjected to grazing had very significantly higher bacterial acvitivity than those never exposed to protozoa ($F_{1,6} = 3964$, $.005 > p > .001$).

In another set of experiments in which protozoa and bacteria were added at the same time, a high number of CPM was observed with the residual protozoa, indicating that different species of bacteria may colonize and/or metabolize at different rates. However, even after subtraction of the CPM associated with residual bacteria, the CPM in grazed stubs were 45% greater than on ungrazed stubs of bacteria (5 SS).

The last set of experiments which employed hypotrichs derived from decaying Thallasia leaves had major problems. On the day we ran the respiration tests (approximately one week after initial incubation), we discovered that the hypotrich culture was contaminated with microflagellates. Therefore, in the separation procedure using the 5 um mesh netting, the microflagellates passed through into the "bacterial" phase, the filtrate. Microflagellates can also feed voraciously on bacteria, and in the present study, they probably multiplied during the incubation period with bacteria. What we thought were controls with bacteria alone really had bacteria-feeding flagellates associated with them. Consequently the respiration (CPM) on these stubs was not

different from those which were subjected to hypotrich grazing. This may indicate the importance of microflagellates on activities of bacteria on surfaces.

Tc show the distribution of bacteria on the surfaces of the stainless steel, the metal stubs were incubated with a mixture of bacteria from the hypotrich cultures, and a series of photographs was taken after the surface was stained with acridine orange. The metal had microscopic ridges and troughs on the surface, and Figure 1 demonstrates that bacteria were present in both, therefore occurring in different planes of focus. Perhaps bacteria in troughs were protected from predation by the protozoan predators, and their numbers multiplied during incubation with the predators.

Figure 2 is a line drawing of a typical hypotrich ciliate, revealing the ventral fused cilia, which these organisms use to "crawl" on surfaces and to sweep bacteria into the oral apparatus.

DISCUSSION AND CONCLUSIONS

The basis for considering that protozoan grazing activities may enhance bacterial acvities on metal surfaces comes from observations of such interactions on natural substrates. It is believed that grazers maintain the bacterial populations in a state of "physiological youth" and prevent the bacterial populations from becoming self-limiting. Enhanced rates of microbial degradation of organic matter have been attributed to grazing by bacteriovorous protozoa with associated detritus (Fenchel, 1970, 1977; Fenchel and Harrison, 1976; Harrison and Mann, 1975; Sherr, et al., 1982). Fenchel (1977) hypothesized that grazing protozoa may enhance bacterial decomposition of detritus by selecting for more rapidly growing bacteria among a mixed assemblage. Sherr, et al., (1982) have shown that availability of phosphorous may limit the breakdown of Peridinium thecae, and that addition of bacteriovorous microflagellates enhances the decay. They speculated that by cropping bacterial biomass, protozoan predators may remove competition for phosphorous. Several investigators have found that the rate of sewage decomposition is facilitated in the presence of ciliated protozoa, and this is considered to be an indirect effect of their feeding on the microorganisms (Butterfield, et al., 1931; Javorncky and Prokesova, 1963).

Mechanical activities of grazing amphipods on detritus have been shown to increase the decomposition process of microorganisms associated with detritus (Fenchel, 1970). Hunter (1980) demonstrated that snails grazing on artificial substrates increased the chlorophyll a level of algal populations. The increase in chlorophyll was attributed to the

grazing which kept the trunover rate so high that few cells became senescent.

Results of the present study strongly indicate that at least certain protozoan predators of bacteria may influence both numbers and activities of bacteria associated with metal surfaces in seawater. One of the reasons for such interactions may lie in the nature of the metal surface itself. As mentioned above, the bacteria were observed in microscopic troughs scratched into the surface. These bacteria may not be available to the predators. They may escape predation, and flourish on the available nutrients which may include metabolites of the predators.

The project was riddled with difficulties which caused us to try new approaches several times. Suggestions for future research in this area, based on results and observations from the present study will be discussed below. Overall, the data suggest the following:

1. Numbers of bacteria associated with metal surfaces may be increased by the grazing activities of protozoan predators of bacteria.
2. bacterial populations associated with metal surfaces may be more metabolically active on metal surfaces in the presence of grazing protozoa.
3. The time of arrival of the predators in relation to the establishment of a pioneer bacterial community on metal surfaces may influence the effects observed in 1 and 2 above.
4. The type of bacteria may influence results observed in 1 and 2 above.
5. Competition between protozoan bacteria-feeders may influence results observed in 1 and 2 above.

The data and problems of the project have led us to consider a field approach for future investigations of the effects of protozoa and higher organisms on activities of bacteria on surfaces of metals. The current project provided a good preliminary look at the possible effects, however, a number of laboratory-imposed conditions restrict us from making solid predictions regarding events in a natural environment. For example, hypotrich cultures contained bacteria closely associated with the protozoa, and these bacteria were introduced in the experiments as contaminants. We may have been able to "axenize" the hypotrichs (though possibly not) by incubating them in combinations of antibiotics. This would have been so unnatural, that it may be meaningless, therefore, we decided to allow the bacteria to be present, but account for their participation by establishing appropriate controls. Likewise, the use of a single strain of bacteria at a time for colonizing the metal surfaces is also unnatural, and that was the reason we attempted to try the community of bacteria originally associated with the hypotrich cultures.

A field approach to determining the effects of predators of bacteria on bacterial actvities has many advantages over the laboratory study, and the following will compare some of these advantages with our laboratory methods:

1. Natural fouling succession— Many different types of interactions among organisms may occur which cannot be simulated in laboratory models. The development of a bacterial film and arrival of predators (in controls) will proceed in a natural succession in the field, whereas in the lab, we introduced the predator species (except in one case) after a given period of time, and this may be very unrealistic, because

the predators may be present with the bacteria in the environment surrounding the metals.

2. Natural population densities-- It is very difficult to enumerate certain species of protozoa in nature which closely associate with but do not securely attach to surfaces. Therefore, we have no accurate estimation of the numbers of these species to introduce into laboratory experiments. Natural concentrations of these organisms would be present in a field study, and results of their effects may be very different from what we found in the lab.

3. Organisms difficult to culture-- There are a number of protozoan and bacterial species which cannot easily be cultured in the lab. In fact, many native bacteria are unculturable, yet they may be very active in the natural environment. Recently we were not successful in obtaining marine amobae, for example, although we are certain they are present and feed on bacteria of surfaces in a marine environment. Other types of protozoa are sessile, such as Zoothamnium and Folliculina, which may not necessarily feed on surface films, but on bacteria in suspension, and may affect the attachment of bacteria from the surrounding environment. Additionally, these relatively large sessile protozoa may com ete with bacteria for surface area, as it is not known whether they require a fouled surface prior to their attachment. Both of these species have been shown to be present on stainless steel after 15 days of exposure to a marine environment (Berk, et al, 1981; Walch, pers. comm.). These organisms are quite common in marine environments, yet Zoothamnium is difficult to culture. To date, we know of no success in culturing this protozoon. For lab experiments, it would take a longer time to acquire adequate numbers of the larger sessile forms than the smaller

bacteriovores.

Although results of the current project suggest that certain protozoa may influence bacterial activities on metal surfaces, implying that they can indirectly influence corrosion rates, there probably would be no point in pursuing it further with a laboratory approach for reasons discussed above. The project provided a foundation for future studies, and the data are being prepared for publication.

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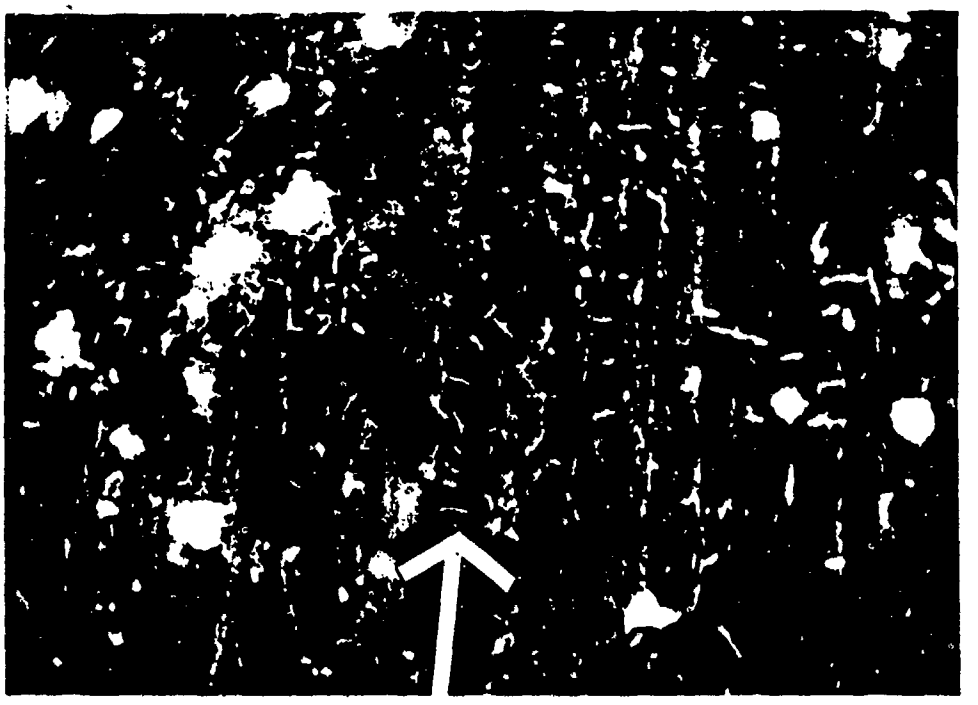
Figure 1. Colonized surface of 316 stainless steel. Note the different planes of focus indicating that bacteria colonize within the microscopic troughs in the surface. The arrow in each photograph points to the same spot on a metal stub, except that the plane of focus is different to reveal the troughs and surface of the metal. A. Bacteria in focus within a trough. B. Bacteria in focus in a plane above the trough.

FIGURE 1.



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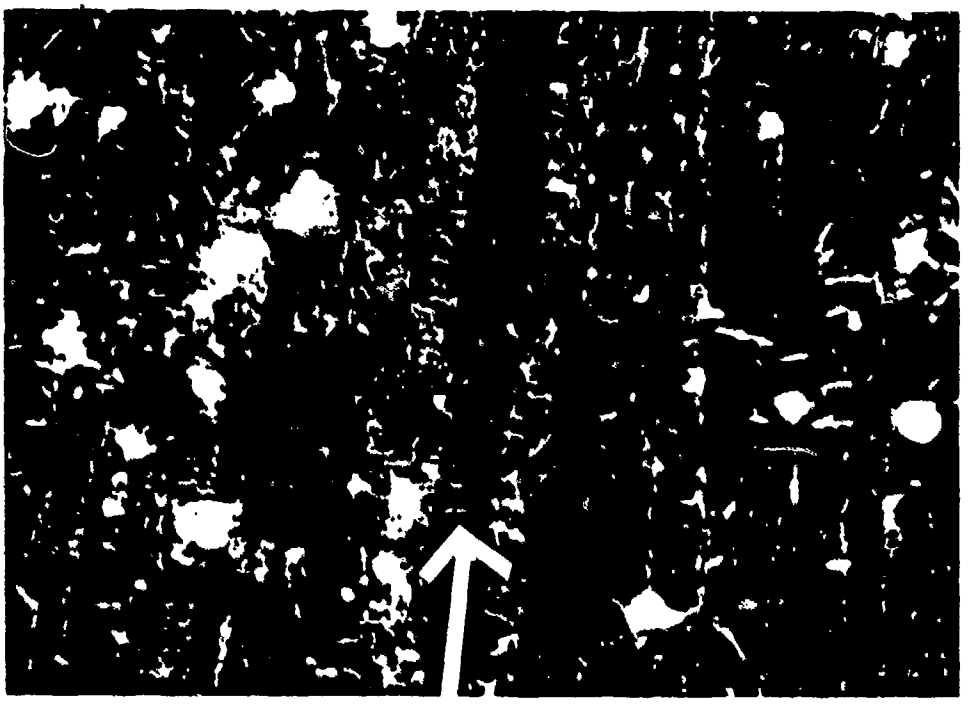
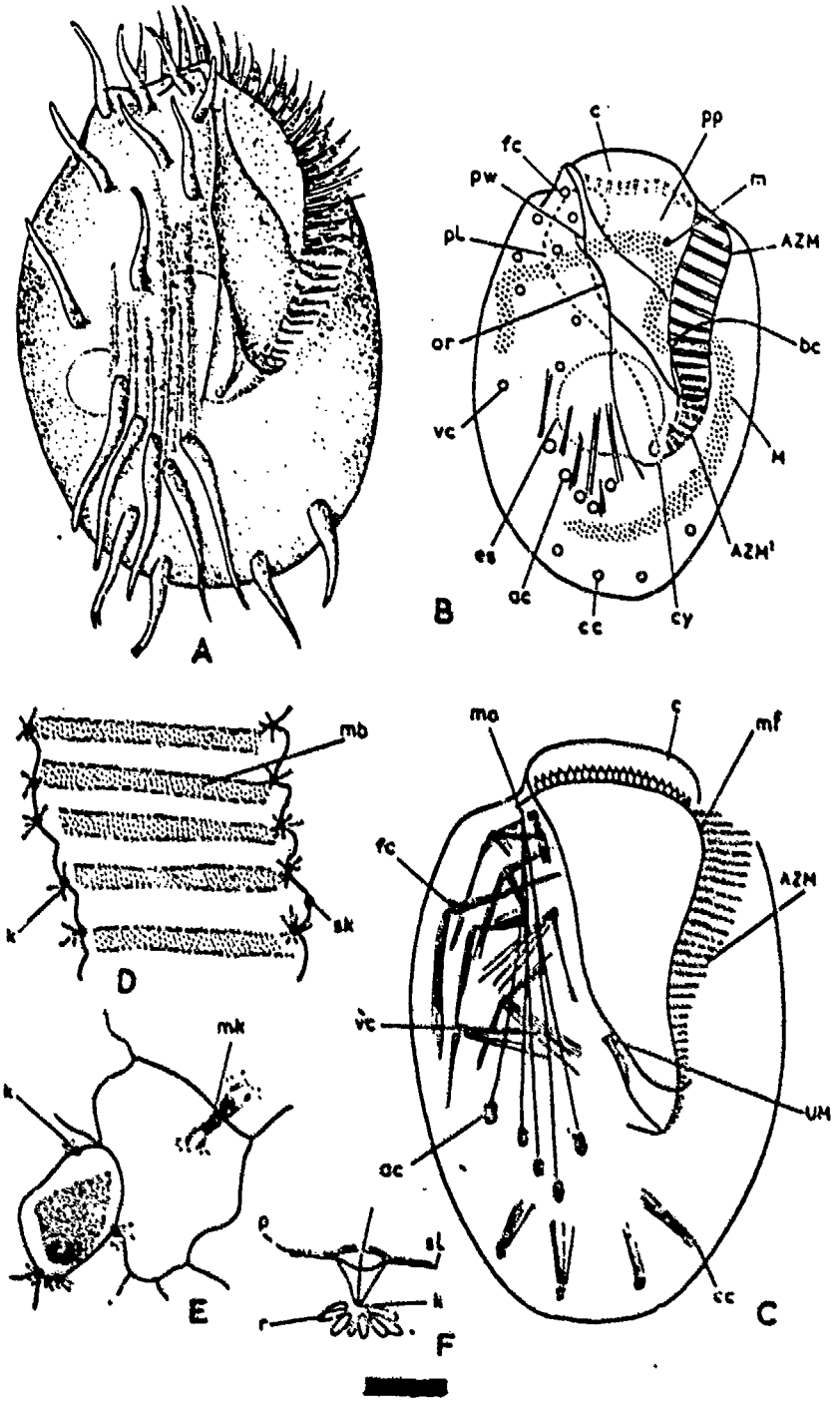


B.

Figure 2. Drawings of several species of hypotrichs (from Mackinnon and Hawes, 1961), showing the fused cilia on the ventral surfaces. These organelles are used to move the organisms across surfaces much like legs of insects, and certain cirri are used to sweep particles to the oral area.

FIGURE 2.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